EXHIBIT A

COLLAGEN

Volume III
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Editor

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I. INTRODUCTION

Collagen fibers constitute the fundamental structural framework of our tissues and organs. Collagen is also the single most abundant protein, and accounts for around 30% of all proteins present in mammals.[1] Collagen is deposited rapidly during periods of rapid growth, and its rate of synthesis declines with age particularly in tissues that undergo little remodeling. The intracellular synthetic process, which leads to the synthesis of procollagen, is relatively complex since it involves a series of posttranslational modifications.[1] The catabolic pathway is modulated by the activity of the enzyme collagenase. In between these two extreme events, there is a process of maturation and cross-linking which provides the collagen fibrils with mechanical and biological stability. The collagen molecules can be extracted from tissues and reconstituted into fibrils or the tissues themselves (heart valves, tendons, pericardium, etc.) can be cross-linked and stabilized for use as bioprosthesis. It is this concept that prompted us to attempt to use such tissues as well as various forms of reconstituted collagen, collagen composites, or modified collagen products as bioprosthetic replacements.

Our own interest in this area began in 1966 following our observation that glutaraldehyde could introduce stable cross-links into collagen fibers devoid of native cross-links.[2] Other aldehydes tested, such as formaldehyde, were not as effective, nor did they generate the thermally and chemically stable cross-links introduced by glutaraldehyde.

The practical application of this concept in our laboratory was prompted in 1969 by Warran Hancock who suggested that the porcine heart valve could, if properly handled, provide suitable prosthetic replacements for diseased tissues. Aware of earlier failures of formaldehyde to accomplish this task, we decided to use glutaraldehyde.[3,4] A very simple experiment was devised to rapidly test a variety of agents for their ability to stabilize the collagen framework of vascular tissues. Porcine aortic valves, with surrounding muscular and connective tissues, were fixed in a variety of reagents which included formaldehyde, crotonaldehyde, acetaldehyde, and glutaraldehyde, and other aldehyde analogues under various conditions of ionic strength, pH, and temperature. Tissues were transferred to a large container and exposed to running water at room temperature. Most tissues began to disintegrate rapidly. After a few weeks, the only tissues that failed to show macroscopic and microscopic structural degeneration were those treated with glutaraldehyde at neutral pH.

These rather simple and direct observations stimulated us to develop more refined tests to quantitate the physical, chemical, and biological stability of the cross-links introduced by glutaraldehyde into collagen. Various testing procedures were designed, which confirmed the above studies.[5-7] Biomechanical studies, heat of denaturation and associated shrinkage, enzymatic degradation, etc. proved the cross-linked material to be satisfactory and encouraged the implantation of the first such valves fixed with glutaraldehyde later that year into humans.[8-10] Similar studies were being conducted independently by Carpentier and associate in Paris, which resulted in the implantation into humans of the first glutaraldehyde cross-linked prosthesis.[11]

The success that followed when cross-linked xenografts generated functional heart valve

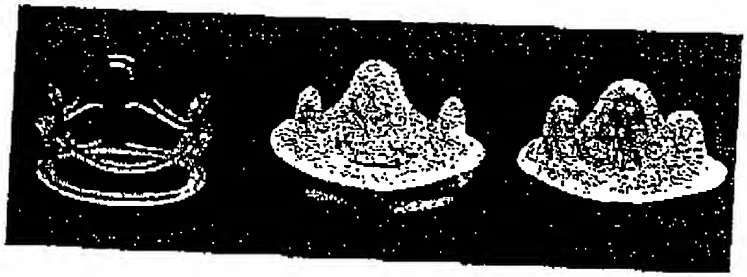

FIGURE 1. Basic components used to assemble one of the early versions of a Hancock valve: a machine tooled polypropylene stent, a sewing ring, and a glutaraldehyde cross-linked porcine aortic valve.

replacement stimulated considerable interest in this technology. This approach is now applied successfully to other prosthetic materials such as skin, tendons and ligaments, pericardial patches, collagen implants, etc. Many interesting findings and several problems have arisen over the years of follow-up of patients with such collagen derived implants. These include fibrotic infiltration, matrix degeneration, calcification, immune rejection, sensitization, toxicity of unreacted glutaraldehyde, thrombosis, and infection. These events have stimulated our laboratory as well as many others to look at various aspects of the chemistry and biology of the implanted material and the host response.

Our earlier studies were followed by attempts to understand the chemistry and reactivity of glutaraldehyde with model compounds and monomeric and polymeric collagen.[12-14] Concomitantly, we continued to investigate the biological compatibility of glutaraldehyde cross-linked collagen with cells, tissues, and body fluids while we attempted to chemically modify the surface of the fibrils and covalently attach macromolecules to the insoluble matrix.[15] In particular, we have been interested in serum proteins (albumin) in glycosaminoglycans (chondroitin sulfate) and diphosphonates (3-amino-1-hydroxypropane-1,1-diphosphonic acid or APD) as a means of preventing or inhibiting the calcification of collagen implants, a major reason for failure of the devices. A variety of in vitro nucleation and calcium uptake tests were adapted to this purpose, as well as implant studies in animals. The compatibility of glutaraldehyde cross-linked collagen with cells and tissues has been investigated using tissue culture techniques and subcutaneous implantation into rats of different ages. The blood-surface interphase was studied using scanning electron microscopy, platelet aggregation, and ATP release assays. The biological stability of cross-linked tissues and reconstituted collagen fibrils and that of chemically modified preparations was investigated in rats histologically and by in vitro enzyme digestion assays. This enabled us to assess the degree of cross-linking and resistance to biological degradation of the collagen implant. The immunogenicity of cross-linked collagen derived from bovine tissues was investigated in rabbits using procedures developed specifically for these studies to overcome the high degree of insolubility of the cross-linked antigen. The studies summarized in this communication represent the combined efforts of many investigators who shared the common interest of further understanding the chemistry and biology of collagen in relationship to its biomaterial properties. Some of the bioprostheses that have originated from the application of the technology are illustrated in Figures 1, 2, and 3.

II. CHEMISTRY OF GLUTARALDEHYDE

A. Reaction of Model Amino Compounds with Glutaraldehyde

Equal molar ratios of 6-aminohexanoic acid and glutaraldehyde (0.05 *M*) were allowed

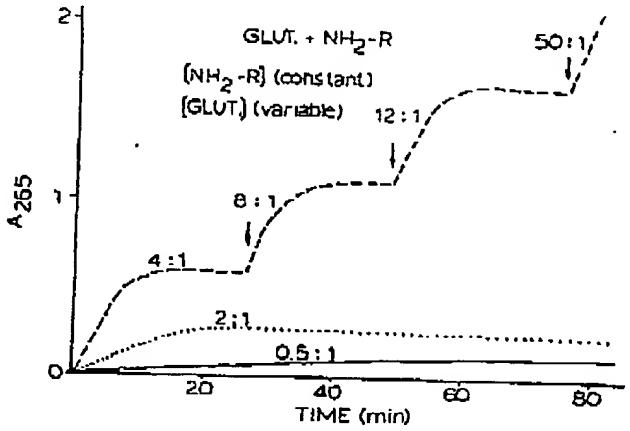

FIGURE 8. Time course of the formation of the 265 nm absorbing products in reaction mixtures containing a constant amount of 6-aminohexanoic acid (0.01 *M*) and increasing amounts of glutaraldehyde: (—) 0.04 *M* glutaraldehyde (4:1), (...) 0.02 *M* glutaraldehyde (2:1), (—) 0.005 *M* glutaraldehyde (0.5:1). Arrows indicate new additions of fourfold or more excess of glutaraldehyde at each indicated time point. Numerical rating reflect the relative molar concentrations of glutaraldehyde:6-aminohexanoic acid at each point in the total reaction mixture.

in the number of lysyl residues modified. This reflects an increase in the molecular length of the glutaraldehyde polymers extending from the initial glutaraldehyde and lysyl residue reaction sites rather than an increase in the actual number of cross-linking sites. These conclusions arise from the observation that after free glutaraldehyde becomes depleted from the solution by binding to reactive groups, subsequent addition of glutaraldehyde molecules causes these to add to those already reacted (Figure 8). When this occurs on the surface of collagen molecules, this reaction will give use to large molecular weight polymers of glutaraldehyde that will now be able to generate "long range cross-links" between further removed reactive sites.

B. Cross-Linking of Collagen in Tissue Matrices

When dealing with fixation of tissues or of densely packed molecules such as collagen fibers, additional variables were introduced. Under these circumstances, penetration of the glutaraldehyde molecules and accessibility to the reactive group on the proteins became a significant issue.[31] When dealing with whole tissues, this becomes a matter of concern even more so. Penetration at room temperature is definitely faster than in the cold. Glutaraldehyde (2%) penetrates into soft animal tissues (i.e., liver) 0.7 mm in 3° hr at room temperature, while its ability to produce adequate fixation lags behind since it reaches a depth of only 0.5 mm in the same period of time. After 24° hr, glutaraldehyde penetrates to a depth of 1.5 mm, while good fixation reaches to a depth of 1.0 mm. However, the maximum penetration of human liver by 4% glutaraldehyde in 24° hr at room temperature and in the cold was 4.5 mm and 2.5. mm, respectively.[32] Other investigators have also presented data on the penetration rate of glutaraldehyde into rat liver.[33-35] A mixture of glutaraldehyde (2%) and formaldehyde (2%) was shown to penetrate human liver to depths of 2.0, 2.5, and 5.0 mm in 4, 12, and 24° hr, respectively.[35]

This relatively slow rate of tissue penetration and the uncertainty of its degree of reactivity as the distance from the surface increases, can cause problems when fixing tissues for electron